REPEATED AGGLUTINATION TESTS BY THE DREYER METHOD IN THE DIAGNOSIS OF ENTERIC FEVER IN INOCULATED PERSONS

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For the diagnosis of enteric fever (typhoid and paratyphoid A and B fevers), both agglutination tests and cultures from blood, urine or stool have long been considered indispensable. A positive culture from any of these sources is of course absolute proof of the existence of active infection (or of carrier in the case of the urine or stool), and should be tried in doubtful cases as many times as possible; but unfortunately, it has not proved possible under usual clinical conditions, to obtain positive results in a very high percentage of cases. In special investigations, to be sure, positive blood cultures have been obtained in two-thirds of all cases tested,[1] or in even more if taken early in the disease; positive urine cultures in about 25% of all patients[2] and positive stool cultures in from 10-81% of cases,[3] the higher figures being obtained late in the disease. It is widely admitted, however, that the routine results even of the best clinical laboratories fall far short of these favorable figures, and it must now further be recognized that under certain unfavorable conditions, even less satisfactory cultural results may be expected. One such condition is the great prevalence of antityphoid inoculation, which not only distorts the accepted clinical picture of this already "protean" disease, but which a priori should make positive cultures more difficult to obtain. The best evidence on this point (from unpublished statistics) indicates that positive blood cultures are only obtained with half the frequency in inoculated individuals (11 vs. 22%), but the figures for positive urine and feces cultures are about the same in both inoculated and uninoculated. In military service, in which the proper diagnosis of enteric fever is today of prime importance, the exigencies of active

[1] Coleman and Buxton: Am. Jour. Med. Sc., 1907, 133, p. 896.
[2] Hiss and Zinsser: Textbook of Bacteriology, 1916, p. 411.
[3] Hiss: Medical News, 1901, 78, p. 728.

service, with the necessarily frequent removal of patients and lack of adequate facilities near the front, beset the cultural diagnosis with still further difficulties. One or more cultures should always be taken from the blood, however, if the case is seen early; or from the urine or stool, if late in the course of the disease.

The importance of agglutination tests, therefore, would be at once obvious, were it not for the difficulties that antityphoid inoculation here also presents. Former methods of making a diagnosis after a single and but slightly quantitative test have now had to be abandoned as worthless, because, as is well known, specific agglutinins are present for many months in the blood of inoculated individuals. As the agglutinin titer after infection or inoculation follows a definite curve, however, it is possible to determine whether repeated tests in a given case follow the steady line resulting from a more or less remote inoculation or the sharp variation of a recent and still active infection. As Glynn[4] has recently pointed out, "the necessity of repeated serologic tests in soldiers suspected to have typhoid, but previously inoculated against it, was suggested in India in 1910 by officers of the R. A. M. C.,[5] Dreyer,[6] Walker[7] and others should also be credited with emphasizing the need of repeated tests, and simplifying and standardizing the technic. It has been shown that whereas agglutinin formation does not occur until the 2nd week of the disease, a sharp rise then occurs, which reaches its maximum between the 16th and 24th day. (Both Glynn and Dreyer have found typhoid agglutinins in dilutions as high as 1:200,000.) (It is well to note that in abortive cases this maximum may not be reached until after the temperature has returned to normal.) This is followed by a sudden drop usually lasting several days, which tapers off into a curve that gradually drops for several months to zero.

The diagnostic value of repeated tests by Dreyer's method, it is the purpose to examine in the present paper, pointing out some of its limitations and dangers, and including the results of some experiments on healthy inoculated individuals. As at present practiced in the British Army, the test is made by agglutinating the patient's serum against "Standard Agglutinable Cultures," prepared solely by the

[4] Glynn, and others: Report on 2,360 Enteritis Convalescents, Med. Res. Com. Spec. Series, 1907, 7, p. 48.

[5] Annual Reports of Divisional Sanitary Officers and Enteric Depots, 1910, Simla.

[6] Proc. Roy. Soc. Med., 1915-1916, 9, p. 11, Med. Sec., p. 1.

[7] Lancet, 1915, 1, p. 324.

Department of Pathology of Oxford University. In addition to the greater ease and economy of this centralization of culture preparation, it theoretically allows results obtained in one laboratory to be compared with later results on the same patient that may have been obtained in a different laboratory. As will be shown later, however, the many factors that enter into the final reading play such an important part that results from different laboratories should only be relied on to a certain — or rather to a somewhat uncertain — degree.

NOTES ON TECHNIC

Practical experience with the method indicates certain phases in the procedure that should be dealt with in detail:

1. The person obtaining the blood should be sure of getting a liberal supply. More than twice as much as for the microscopic test is needed to secure the necessary 6 drops of serum. If insufficient blood is obtained, or Dreyer's pipet is not at hand, an alternate "volume" method with an ordinary pipet and rubber teat may be employed. In this method the available serum is drawn up to a grease or file mark on the pipet, and a 1:10 dilution obtained by mixing with 9 volumes of diluent drawn to the same mark. An appropriate amount of diluted serum is now drawn to a mark higher on the pipet, discharged into tube 1 of the series and mixed with an equal amount of water. One half of this dilution (i. e., to the higher mark) is then withdrawn, deposited in the next tube, mixed with an equal amount of water and so on through the series. The process is repeated with the second and third rows with appropriate cleansing of the pipet and finally an equal amount of the standard agglutinable culture (i. e., to the higher mark) added to each tube. This gives a final dilution of 1:40, 1:80, etc., but the dilution may of course be varied to suit individual cases. The grease marks should be high enough on the pipet to ensure that the final dilution at least half fills the tube.

2. The agglutination cultures should always be practically transparent (i. e., not contaminated), and should never be acid. It is stated by the Oxford Standard Laboratory that "the growth of a mould in a bottle does not affect the agglutinability of the culture. If the mould be fished out and a drop or 2 of chloroform be added to the fluid to prevent further growth, the culture is as good as ever. Bacterial growths in the cultures also occur, but are rare and almost invariably the result of careless handling." In our experience, however, contaminations of both kinds are common in spite of careful handling, unless a given set of bottles is used up in a few days. To lessen the chance of contamination, the approximately requisite amount is poured into watch glasses for use and the excess discarded. It has been found expedient also to discard all cultures showing signs of contamination. We have found by comparative tests that unaccountably low agglutination readings can usually be traced to spoiled cultures.

3. Although the directions call for dilutions of serum with normal salt solution, it has been the custom with many pathologists using this method to use distilled water instead. It is thought that sharper results are obtained, and we understand that either diluent is permitted by the British authorities. We have

[8] Jour. Path. and Bact., 1909, 13, p. 331.

made some comparisons of readings obtained with salt solution and water, and find that readings of the latter average almost a whole tube higher. Thus in 39 tests of both methods, a total of 28 higher tubes was obtained with distilled water as diluent, when read with the naked eye; of 32 when a hand glass was used. In only 12 were similar readings obtained and in 2 the salt solution gave 1 tube higher reading. We, therefore, feel that only one diluent should be used by every one, and as the flocculi seem more distinct with distilled water, would prefer the use of the latter.

4. Another variation in technic which is not only considered permissible, but is practiced by many users of the method, is the use of a hand glass in making the readings. We have compared readings in over 400 tubes, using a hand glass of 2 diameters, and have found that in the majority of cases, higher readings of from 1-5 tubes are obtained. The difference is less marked with B. typhosus (28 tubes in 43 comparisons), than it is with the paratyphoid (60 tubes in 39 comparisons of A and 61 tubes in 42 comparisons of B). This is probably due to the normally greater size of the typhosus clumps when the Oxford Standard Agglutinable Cultures are used. The reading with the hand glass seems preferable, as it allows detection of true agglutination to its furthest limits. The flocculi have the characteristic appearance, still higher readings are not obtained if a more powerful glass is used, and if the test is allowed to stand over night, the "naked eye" readings approximate the previous "hand glass" readings closer than they do those of the previous "naked eye" readings.

5. If there is any doubt about an individual reading (particularly if in the B group), or if circumstances will permit, it is advisable to let the Dreyer stand wait over night and make a second reading the next morning. If these subsequent readings are higher they should be included in the final report.

6. All tubes of the series should be examined, because it sometimes happens (especially in the paratyphoid series), that the stronger dilutions of serum will not agglutinate as well as the weaker ones. The over-night reading, especially the para B tests, will also obviate mistakes due to this cause.

7. It has recently been suggested that the best results are obtained if the height of the water in the bath is such that one-half of the mixture in the tubes is in and one-half out of water. We have made no tests of the value of this detail. It is convenient to have a water bath of distilled water and in a covered container, so that dirt or calcium deposits on the tubes will not interfere with the reading.

8. If the regulation Dreyer pipet is used it is important that the same pipet or another of the same external caliber should be used throughout the test. Donald[9] has shown that the size of the drop varies directly with the external diameter of the tip. Inasmuch as the drop becomes smaller the more horizontal the pipet is held, the angle at which the pipet is held should be the same throughout the operation. There is no appreciable difference in the size when the pipet is held at 80 or 90° (Donald[10] and Garrow[11]).

9. Other simplications in the use of the Dreyer pipet are included in the following letter from Professor Glynn: "The 6 drops of serum to be tested are distributed from a clean and absolutely dry pipet. The surplus serum is then discharged and the pipet washed out 3 or 4 times with diluent, namely,

[9] Lancet, 1816, 2, p, 423.
[10] Ibid., p. 994.
[11] Ibid., p. 864.

water, 54 drops of which are added to make the dilution of 1:10; there is no use to wash and dry the pipet first. The wet pipet is now used for distributing the water in the small dilution tubes. The surplus water is then blown out of the teat, and by shaking the pipet with the hand any drop on its end is removed. The wet pipet, which now contains inside and out about one-third of a drop of water — this I have ascertained by weighing — is rinsed out in the mixture of serum and water, which is then distributed with it into the small dilution tubes; the addition of a third of a drop of water to 60 is of no practical importance. The pipet is again washed out with water 3 or 4 times, emptied, and shaken as before, and then rinsed out in the bacillary emulsion, which is next distributed in the dilution tubes. In order to save the time of counting the drops of bacillary emulsion it is a very simple matter to calibrate the pipet to hold the right amount by marking the glass with a file. If care is taken to select pipets which are all approximately the same size as measured by a gauge, then one calibrated pipet can always be used for typhoid emulsion only, another for the paratyphoid A, and a third for paratyphoid B. This saves still more time. In fact, instead of washing out the pipet with 3 different fluids and drying on 6 occasions (for a single test against 1 bacillus) it is then only necessary to wash the pipet 3 or 4 times with 1 fluid on 1 occasion to make a single test with 3 different emulsions, excluding the final washing and drying."

TABLE 1

COMPARATIVE AGGLUTINATIONS IN DIFFERENT LABORATORIES

Hospital Number	Patient 1			Patient 2		
	Typhoid	Paratyphoid A	Paratyphoid B	Typhoid	Paratyphoid A	Paratyphoid B
1	1:250	1.500	1:125	1:1000	1:1000	1:250
2*	1:500	1:250	1:125	1:1000	1:1000	1:500
3	1:250	1:50	1:25	1:125	1:250	0
4	1:640	1:160	0	1:80	1:40	0

* A sample of the blood of Patient 2 done the next day at this hospital with different cultures gave T. 1:250; A. 1:250; B. 1:125. Reduced to agglutinin units the differences are slightly greater.

10. The best and most consistent results are naturally obtained when the tests are done by the same observer using the same batch of cultures. If successive tests on a given patient are done in different laboratories, allowance must be made for a greater factor of variation in the method. This is not only due to the "personal equation" that enters into most laboratory tests, and to possible slight variations in the various batches of the standard cultures, but also to the remediable lack of uniformity in the various details just discussed. If, for instance, one observer should happen to use salt solution as a diluent, and make an immediate reading with the naked eye he might easily get a reading 3 tubes lower than another observer using distilled water and reading the next morning with a hand glass (e. g., a difference of 1:25 and 1:250). That this is not an imaginary situation is shown by the following control tests done simultaneously on the same blood by 4 pathologists in adjacent laboratories.

As is indicated by the first sentence in the footnote, it is very probable that variations in the different batches of agglutinable cultures were responsible for at least some of these discrepancies. Such figures do not indicate that comparisons from different laboratories are valueless, especially as in active infection the

rise in agglutinins amounts to even greater differences than are here involved; but they do mean that all steps must be carefully standardized and that even then too much reliance must not be placed on small fluctuations. Differences of 1 tube (e. g., between 1 : 50 and 1 : 125) should hardly be taken into consideration, and in some cases even greater fluctuations do not form sufficient basis for positive diagnosis.

11. The directions for placing "Standard Agglutination" midway between 2 dilutions under certain conditions have been still further amplified in the following interpolation table and factors, depending on the character of the sediment or flocculi as seen by the naked eye.

TABLE 2

DREYER'S INTERPOLATION TABLES AND FACTORS

Agglutination	Factors
Total (i. e. all sedimented)	1.47
Total (mostly sedimented, few flocculi)	1.29
Standard (very large flocculi, slight if any sediment)	1.19
Standard (large flocculi, no sediment)	1.13
Standard	1.00
Standard (flocculi smaller)	0.88
Trace (large numbers of small flocculi)	0.77
Trace (many very minute flocculi)	0.68
Trace (if very slightest granules appear)	0.60
Trace ("a very doubtful tube indeed")	0.53

We have found, however, that the maximum positive readings (with a hand glass, at any rate) are always in the "trace" group. The differences involved are, therefore, so much less than those introduced by other variable factors that the use of these tables only seems to detract from the simplicity of the test without adding to its accuracy. Also a comparison of results obtained with different batches of cultures has given us the impression that the character of the flocculi may vary with different batches, so that for this reason also the soundest policy is to rely only on the maximum dilution in which agglutination is detectable.

INTERPRETATION OF RESULTS

In interpretation of results, a few points in addition to those given under the head of "diagnosis" in the directions are useful. When 3 or more tests have been made and the results translated into agglutinin units, the curve obtained must be interpreted both according to the date of onset and to the date, number and kind of inoculations received. The date of onset should be elicited by careful questioning. If the maximum agglutinin titer apparently falls outside the 16-24 day limit, the date of onset should be again investigated. If it really falls outside, a positive diagnosis should be avoided, even with a rise of 100-200%, as other fevers may cause similar fluctuations. It should also be remembered that 2 successive equal observations may have missed an intervening maximum and that an apparent maximum is rarely the true highest point reached. In allowing for the effect of inoculation,

though each individual's agglutinins respond to inoculation with different intensity, nevertheless, the general direction of the curve is the same in all, so that a low response shortly after inoculation, or a high one many months after, point in themselves to absence or presence of infection, respectively. Of course, if only typhoid vaccine has been given, even low agglutination in either paratyphoid series has diagnostic importance. There is some evidence that other fevers may reduce inoculation agglutinins, but this point still remains undecided and has not been investigated by us. Finally, if, as is the case in the British Expeditionary Forces, the ultimate diagnosis rests with the pathologist, he should give careful consideration in consultation with the clinician to the clinical aspect of the case. It has been found advisable and very useful to make use of the term "Enteric Group" for those cases whose dates are very suggestive, but not sufficiently concise to warrant the more accurate diagnosis of any one of the three.

AGGLUTININ CURVES IN T. A. B. INOCULATED INDIVIDUALS

Advantage was taken of recent T. A. B. inocnlations in previously inoculated individuals to study the resulting agglutination curves. In the following charts are given the average curve (composite agglutinogram) obtained from 40 such individuals, also an example of one of the maximum and one of the minimum responses. As the former had been proved not to be a carrier by repeated negative stool examinations, and as neither were isolated instances, they may be taken as showing the marked variations of individual response to inoculation that may be expected. The series were also tested one month after inoculation, but as the results averaged lower than in the following month (i. e., obviously incorrect) they were not included. They are mentioned here to show how incorrect may be the results obtained by those not sufficiently familiar with the methods and its pitfalls. The high but quick falling B. curve, the lower and steadier A. curve, and the intermediate T. curve are similar to results obtained by other observers (Garrow[8]). Lack of time prevented further elaboration of the curves, but they would doubtless have dropped slowly for many months. Garrow, for instance, found an average T. agglutination of 1:40 seven months after inoculation; and of 1:20 sixteen months after.

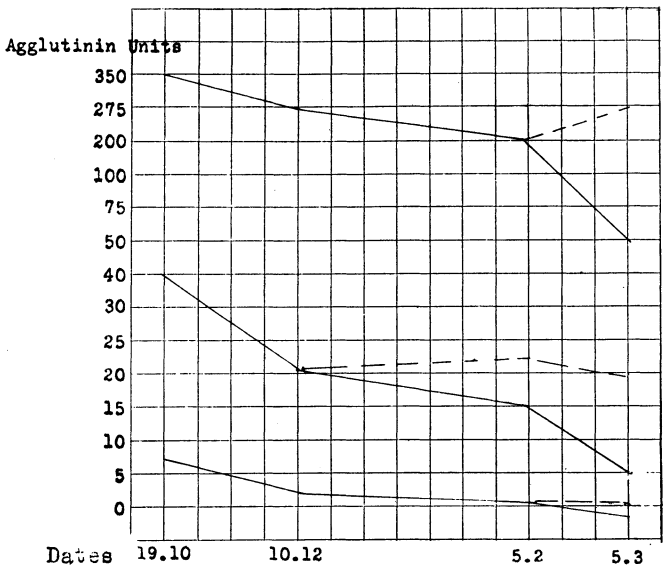

Chart 1.—B. typhosus agglutinograms after T. A. B. inoculation. In this and the following 2 charts, an average curve of the agglutinin units of 40 individuals is given, also sample curves of high and low responses. Three doses of U. S. Government vaccine were given at 10-day intervals 2 months before the first test recorded. The usual dose of 0.5, 1, and 1 c c were given, each c c containing 1,000 million typhoid and 750 million of each paratyphoid bacilli. In all charts dotted lines indicate "hand glass reading."

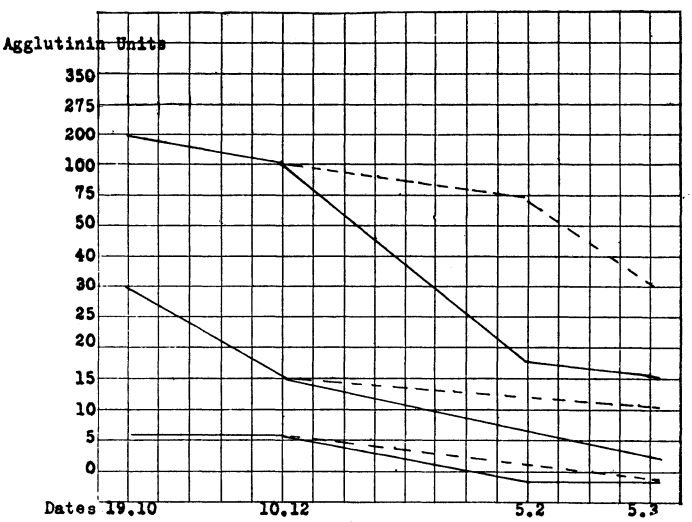

Chart 2.—B. paratyphosus A agglutinograms after T. A. B. inoculation. Same details as Chart 1.

EFFECT OF PARA B INOCULATION IN T. A. INOCULATED INDIVIDUALS

As some of the personnel of this unit had received only typhoid and paratyphoid A inoculation in the spring of 1917, an opportunity was offered when they received para B inoculation in January, 1918, to study the effect of the latter on the T. and A. agglutinins. The accompanying charts show that in all but one case a very distinct but evanescent rise of typhoid agglutinins was produced, whereas the changes in the para A agglutinins were so insignificant that they are

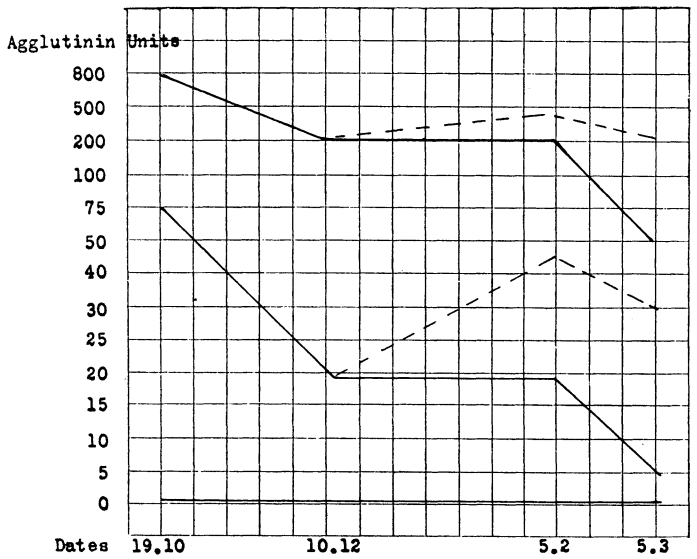

Chart 3.—B. paratyphosus B agglutinograms after T. A. B. inoculation. Same details as Chart 1.

well within the limits of error of the method. This corresponds with other experiences that the agglutination reaction with para A tends to be erratic. From these and similar observations of other observers, it is proper to deduce that paratyphoid fever, like other less related infections, will probably cause a temporary rise in the inoculation typhoid agglutinins (partial, minor or meta agglutinins), and proper allowance must be made for this in the interpretation of agglutinin curves of suspected enteric fevers. Dreyer, for instance, has found that "if an animal inoculated some weeks before with a micro-

organism, be inoculated with a nonlethal dose of vaccine of another kind, its agglutination titer for the first rises and preserves the same course as the original again." Garrow also showed that 60 of 98 typhoid inoculated individuals showed an increase of from 300-6,300%, 10 days after para A and B inoculation. It is interesting also to note that the highest rise in typhoid agglutinins occurred in the test made 3 days after the second inoculation, and 13 days after the first; in other words, that the first inoculation exerted the most stimulating influence and that thereafter (as it was then too early for the second inoculation to have had effect) the agglutinin titer dropped in spite of the next 2 inoculations.

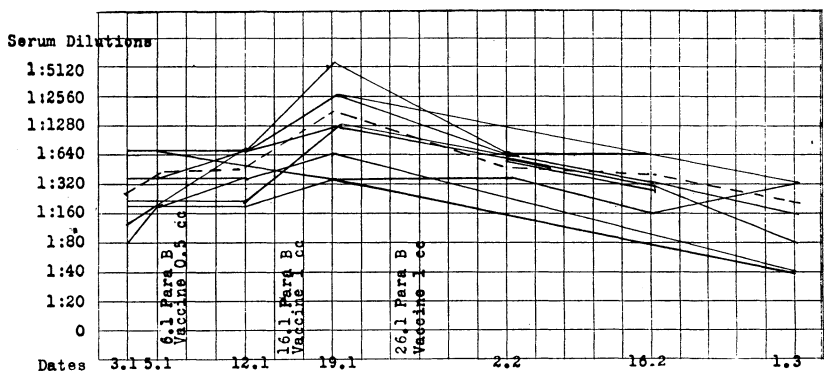

Chart 4.—B. typhosus agglutinograms in T. A. inoculated individuals after inoculation with paratyphoid B vaccine. In this and the following 2 charts individual curves of 8 persons and an average curve are given. They all had received T. and A. vaccine separately 8 months before (i. e., a total of 5,000 million in 5 doses) and received 3 doses of B. vaccine (i. e., 2,500 million in 3 doses) at the time indicated on the chart. Note the nonspecific rise in T. agglutinins. As the agglutinin factors were the same throughout these series, the maximum positive dilutions are given instead of agglutinin units, as in the former charts.

The agglutination tests with para B cultures show that whereas no agglutination was detected before inoculation, B agglutinins had begun to appear in half the cases by the sixth day, and the rest by the twelfth day after inoculation. Here again a maximum titer was quickly reached and the curve began to fall in some cases before the third inoculation had been given. This is in the main similar to the experiences of Garrow[12] who considers 5 phases of agglutination after inoculation: (1) A latent period of 4 or 5 days; (2) a rising period of 7 days; (3) a maximum reached on the 12th day; (4) a

[12] Jour. Roy. Army Med. Corps, 1917, 29, p. 412.

rapidly falling period lasting to about the 24th day; (5) a residual period lasting many months.

Only once did the third inoculation have any influence in determining the maximum titer. This differs from Craig's[13] experiences, where after T. A. B. inoculation the highest titer was always reached shortly after the 3rd weekly inoculation. The behavior of the agglutinin

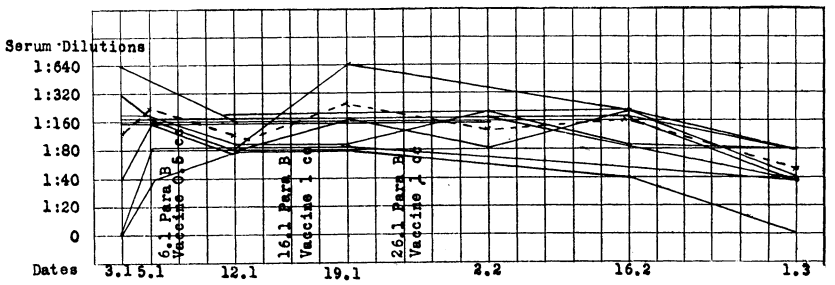

Chart 5.—B. paratyphoid A agglutinograms in T. A. inoculated individuals after inoculation with paratyphoid B vaccine. Note that no appreciable change is caused.

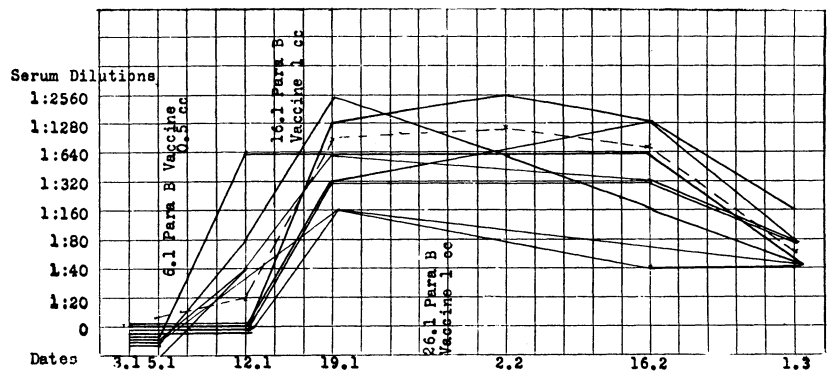

Chart 6.—B. paratyphosus B agglutinograms in T. A. inoculated individuals after inoculation with paratyphoid B vaccine. Note the incubation period and the sharp but partially evanescent rise in agglutinins.

curve during the second and third inoculations might be interpreted either as a depression resulting from the repetition of the dosages, or as such an extreme response to the dose that fell on a virgin field that the responses to the later doses were masked by the decline from the earlier dose. A single opportunity was offered to test this by repeating

[13] Jour. Am. Med. Assn., 1917, 59, p. 1000.

the inoculations at longer intervals. As the curve in this case showed a similar fall to the majority of the earlier cases, it seems as if the latter assumption was correct.

CONCLUSIONS

The diagnosis of fevers of the enteric group may be made by agglutination tests in many cases when cultures have been negative, but the test should never be used as a substitute for cultures.

In inoculated individuals the diagnosis may be made by quantitative agglutination tests, if 3 or more tests are made at suitable intervals and the resulting curve interpreted in the light of the inoculation and clinical data and the date of onset of the disease.

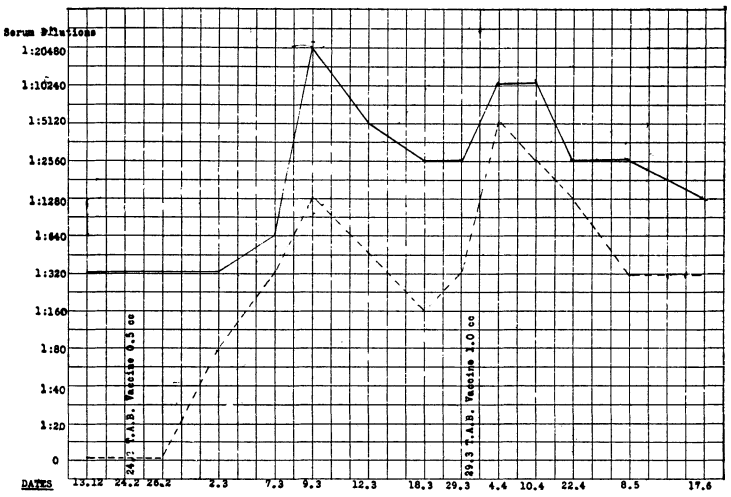

Chart 7.—B. typhosus and Para A agglutinograms in T. A. inoculated person after 2 doses of T. A. B. vaccine.

The Dreyer macroscopic method used with standardized agglutinable cultures not only gives excellent results, but allows comparison to be made of results obtained at different times in different laboratories. These results should be expressed in agglutinin units.

For proper comparison, every step in the method should be standardized; in other words, all workers whose results are to be compared should perform each step in the same way. The most consistent results are obtained by one worker using the same batch of cultures.

Results from different laboratories must be interpreted with caution and allowance made for the personal equation as well as for

possible variations in the cultures. Variations of one or sometimes even two tubes should be disregarded as the changes caused by active infection are much greater than this.

It is recommended that distilled water should be uniformly used as a diluent, and that readings should always be made with a hand glass (4 diameters) in the illumination advised by the Oxford Standard Laboratory.

If standard agglutinable cultures are used, they should be carefully guarded against contamination. If contamination occurs, especially if they have become acid, they should be at once discarded.

In cases in which agglutinations show a marked rise but do not point decisively to any one of the three members of the enteric group, and cultures are negative but clinical evidence suggestive, a diagnosis of "enteric group" should be made. This rise in titer in more than one species is probably due to nonspecific stimulation (i. e., minor or partial agglutinins) or rarely to coagglutination or multiple infection.

We present herewith figures showing the gradual fall in T. A. B. agglutinins in 40 inoculated individuals. If the presence of agglutinins may be taken as an index of protection, these and similar tests show that the methods of prophylactic inoculation now in vogue protect the average individual for more than a year. There is, however, considerable variation in the individual response, and apparently also in the effect of different batches of vaccine.

We also present figures to show that paratyphoid B inoculation in persons who have already received typhoid and para A vaccine, causes a distinct rise in the typhoid agglutinin curve. This fact should be borne in mind in interpreting clinical tests.

A single experiment is given which tends to show that as compared with the American method now in vogue, more efficient prophylactic inoculation may be obtained by giving two (or possible even single) doses at more frequent intervals.

NOTE.—Since this paper was completed an article by Fennel[14] on the same subject has come to hand. The reader is referred to it with the endorsement of the suggestions offered. Various examples of interesting results obtained with the Dreyer method have recently been published by Perry.[15] Other recent investigations by Dreyer and others[16] tend to support our view that prophylactic inoculations would be impaired by lengthening the interval between doses, and giving fewer doses, but repeating the process more frequently.

[14] Jour. Am. Med. Assn., 1918, 70, p. 590.
[15] Lancet, 1918, 1, p. 593.
[16] Ibid., p. 498.